

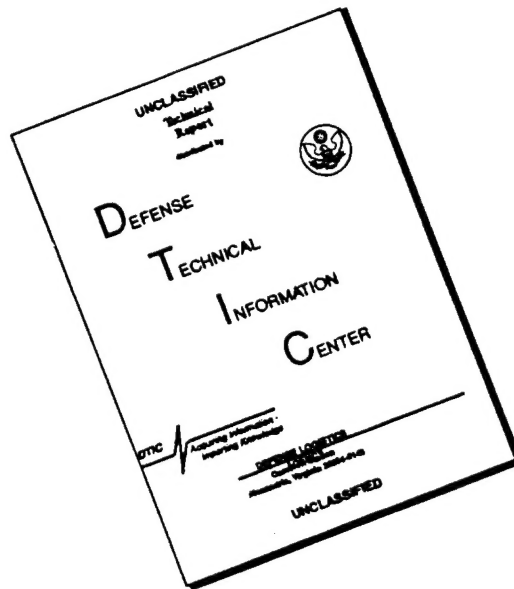
# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Please reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Service, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302 and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 30 September 1996	3. REPORT TYPE AND DATES COVERED Final Technical Report: 1 Aug 94 - 31 Jul 96	
4. TITLE AND SUBTITLE CIRCADIAN RHYTHMS IN ZEBRAFISH			5. FUNDING NUMBERS F49620-94-1-0314 61102F 2312-CS	
6. AUTHOR(S) Joseph C. Besharse, Ph.D. and Gregory M. Cahill, Ph.D				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Kansas Med. Center 3901 Rainbow Blvd. Kansas City, KS 66160 University of Houston (Subcontractor) 4800 Calhoun Rd. Houston, TX 77204			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Air Force Office of Scientific Research/NL 110 Duncan Ave Bolling AFB, DC 20332 Dr Haddad			10. SPONSORING/MONITORING AGENCY REPORT NUMBER AFOSR-TR-96 0452	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)  The goal of this project was to determine whether the zebrafish would be a useful system for genetic analysis of the cellular and molecular mechanisms that comprise vertebrate circadian clocks. An understanding of biological clock mechanisms will aid in the treatment of the performance deficits, sleep disorders and other problems associated with jet lag, shift work and organic clock deficits in humans. Experiments were performed to characterize behavioral, physiological and molecular circadian rhythms that can be used to screen for genetic clock mutations and to investigate the roles of mutant genes. Robust circadian rhythms of behavior in both larval and adult zebrafish were found and characterized. In addition, the exceptionally strong circadian rhythms of melatonin release by cultured zebrafish pineal glands were characterized. The results indicate that genetic analysis of vertebrate clock mechanisms will be feasible using this model system.  UNCLASSIFIED//FORN DISSEM				
14. SUBJECT TERMS			15. NUMBER OF PAGES 10	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT U	18. SECURITY CLASSIFICATION U	19. SECURITY CLASSIFICATION OF ABSTRACT U	20. LIMITATION OF ABSTRACT U	

# DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

## Summary

The goals of the project were to characterize several features of the zebrafish circadian system, to determine whether genetic analysis is feasible, and to develop the methods necessary for identification and characterization of zebrafish clock mutants. The 5 specific aims involved characterization of zebrafish circadian rhythms at three levels of organization: 1) clock organs in vitro, 2) intact individuals in vivo, and 3) populations. We have made significant progress at all levels. Completed studies are summarized below and described in the publications listed. In addition to 2 papers and 3 abstracts listed below, we plan to submit 2 more manuscripts from this work by the end of 1996. Data for those manuscripts is described below.

## Publications

- Cahill, G.M. 1996a. Circadian regulation of melatonin production in cultured zebrafish pineal and retina. *Brain Research* **708**:177-181.
- Cahill, G.M. 1996b. Circadian melatonin rhythms in cultured zebrafish pineals are not affected by catecholamine agonists. In press. *Gen. Comp. Endocrinol.*

## Published Abstracts

- Cahill, G.M., Hurd, M.W. and Debruyne, J. 1996. Zebrafish circadian rhythms: studies of locomotor activity and cultured retina and pineal. *Biol. Rhythm Res.* In press.
- Cahill, G.M. 1995. Pineal function in zebrafish: a model system of circadian rhythmicity. *Biol. Rhythm Res.* **26**: 374.
- Cahill, G.M. 1994. A circadian oscillator in cultured zebrafish pineal regulates melatonin release. *Mol. Biol. Cell (Suppl.)* **5**: 270a (ASCB abstract).

## Manuscripts in preparation

- Hurd, M.W., DeBruyne, J., Hurd, L., Straume, M. and Cahill, G.M. Circadian rhythms in zebrafish locomotor activity.
- Coon, S.L., Begay, V., Falcón, J., Cahill, G.M. and Klein, D.C. Regulation of arylalkylamine-N-acetyltransferase mRNA in the pineals of trout and zebrafish.

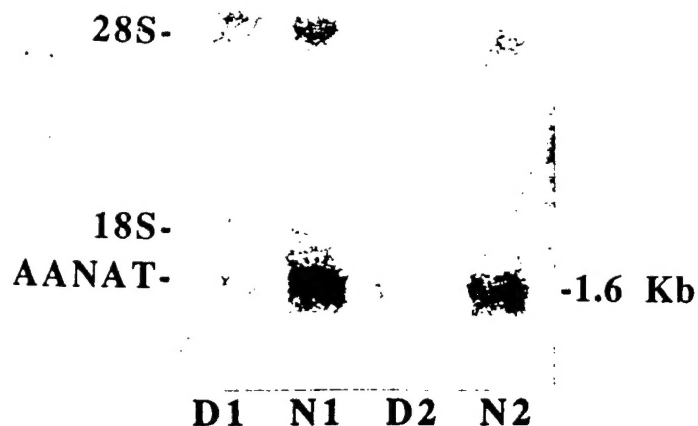
## Progress on Specific Aims:

Specific aim 1. Pineal and retinal melatonin rhythms. We proposed to "develop culture conditions for measurement of melatonin rhythms from pineal and retina and characterize the basic clock properties of these tissues in vitro." Basic characteristics of in vitro melatonin release rhythms are reported in Cahill, 1996a. Briefly, the pineal produces robust circadian rhythms of melatonin release in vitro, and the oscillator can be reset by exposure to shifted light cycles in vitro. The retina produces melatonin rhythms that rapidly damp in culture. Retinal melatonin synthesis can be suppressed acutely by light, but light cycles do not restore rhythmicity. As proposed, we have tested several other culture medium formulations on retinas, with no improvement in rhythmicity.

Pineal and retinal melatonin rhythms in many species are affected in one way or another by catecholamines. We found that catecholamines do not affect zebrafish pineal melatonin rhythms. These data are reported in Cahill, 1996b. We also found that retinal melatonin is suppressed by both dopamine and norepinephrine. Characterization of the receptors mediating melatonin suppression in the retina is in progress.

A cDNA for arylalkylamine N-acetyltransferase (AANAT, the rhythmic, penultimate enzyme in melatonin synthesis) was first cloned last year. In collaboration with S. Coon and D.C. Klein at NIH and J. Falcon at University of Poitiers, France, we are investigating regulation of AANAT mRNA in trout and zebrafish pineals. We have found that AANAT mRNA levels are rhythmic in pineals cultured in constant dark (Fig. 1) and in constant light (not shown). Light inhibits zebrafish pineal melatonin release, and it inhibits AANAT activity in many systems, but we can detect no acute effect of light or darkness on AANAT mRNA. This indicates post-transcriptional regulation of AANAT activity in response to light. We also analyzed the tissue distribution of AANAT, and find that it is present in pineal and retina, but is not detectable in brain, liver or gonads (not shown). We plan to submit a manuscript on this work by the end of 1996.

**Figure 1.** Circadian regulation of AANAT gene expression in zebrafish pineal. Pineals (~30 per time point) were cultured in constant darkness and harvested at midday (D1,D2) and midnight (N1,N2). The blot of total pineal RNA was probed with a trout AANAT cDNA. A 1.6 kb RNA is rhythmic, with increased levels at night. A rhythmic, higher MW transcript may also exist, but has not been resolved from 28S rRNA.



Specific aim 2. Development of rhythmicity. We proposed to “determine the earliest time in zebrafish development that circadian melatonin rhythms can be measured, and whether melatonin rhythms can be measured from haploid larvae.” The pineal forms during the first day of development, and the first pineal photoreceptors (the putative clock cells) are identifiable by 24 h post-fertilization (pf) at the standard temperature of 28.5°C. Retinal photoreceptors differentiate later. Cone photoreceptors begin to differentiate by 48 hr of development. Outer segments begin to develop and the adult cone mosaic pattern is evident by 60 hr. Zebrafish hatch between 40 and 72 hours of development at 28.5°C. We have taken three approaches to measurement of diurnal rhythmicity in late embryos and early larvae: 1) measurement of melatonin in whole-larvae extracts, 2) measurement of melatonin release from embryos in flow through culture, and 3) observations of hatching. Data from whole-larvae extracts indicate that diurnal rhythmicity is present on the third day pf, while data from the other two types of experiments argue against development of diurnal rhythmicity before the third day.

Diurnal rhythms of melatonin-like immunoreactivity are detectable in whole-larvae extracts by the third day of development in fish raised at 26°C in 14:10 LD cycles (Fig. 3A). Whole larvae (30-600 per sample, depending on age) were homogenized, extracted with methylene chloride, and melatonin was determined by RIA. Melatonin was detectable by the third night pf, and levels were low or undetectable during the day. Experiments to determine whether these rhythms reflect authentic melatonin, whether they are measurable on the second day of development, and when they come under the control of a circadian oscillator are in progress. These data from diploid larvae suggest that it may be possible to measure melatonin rhythms from haploids, but this has not been tested yet.

For measurement of melatonin release, embryos were removed from their chorions and placed individually in flow-through culture at a stage corresponding to 24 h of development (temperature was lowered to delay this stage until 38 hours, the time of the second light offset). The embryos were cultured at 21°C (which we have found to cut the overall rate of development in half) in a 14:10 LD cycle. The embryos were superfused with 10% Hanks buffer containing elevated calcium and 100  $\mu$ M 5-hydroxytryptophan to maximize melatonin production. Figure 2B shows that melatonin release increased to detectable levels during the 3 days of culture, but was not rhythmic. This experiment argues (weakly) against melatonin rhythmicity during the developmental stages corresponding to the second day at 28.5°C.

Attempts to measure hatching rhythms were based on the recent finding that hatching of another fish species, the Medaka, is regulated by a circadian clock (J.J. Sohn, personal communication). Rhythmic hatching would make a simple screen for clock mutants. However, we found no evidence that hatching is rhythmic in zebrafish. Zebrafish incubated at 28.5°C hatch between 40 and 72 hours pf. By lowering the temperature after the first 14 hours pf, we slowed development such that hatching occurred between 3.5 and 5.5 days at 21°C and between 5 and 8.5 days at 18°C. At all 3 temperatures, in a 14:10 LD cycle, the time of hatching was normally distributed around a mean, and no rhythm was apparent (data not shown).

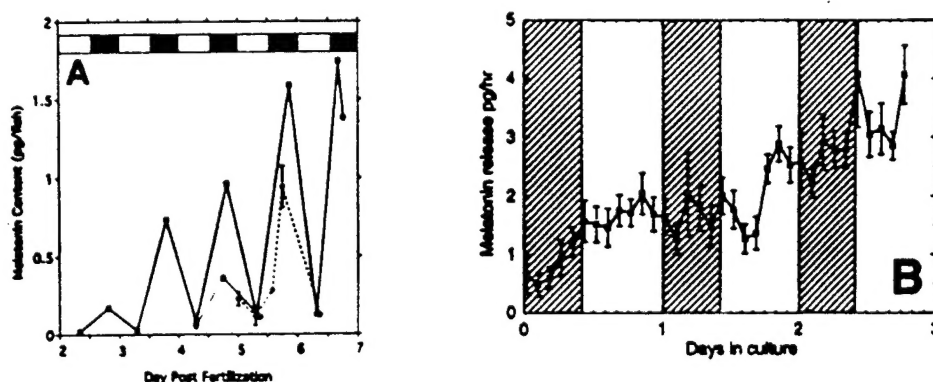


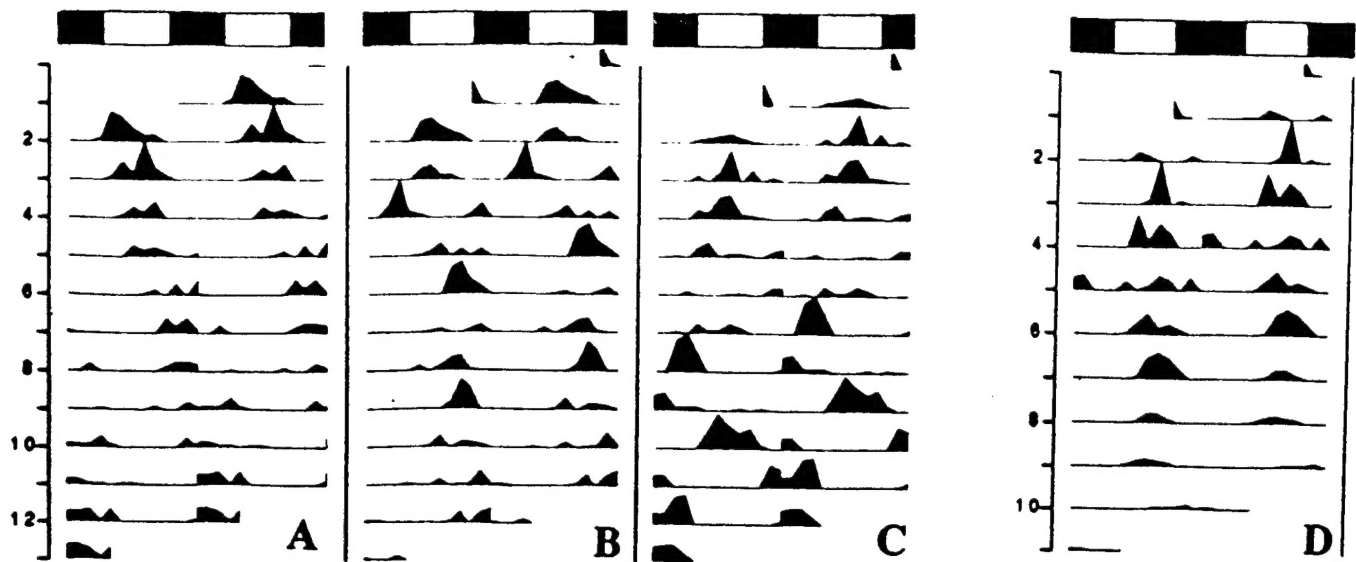
Figure 2. Development of diurnal melatonin rhythms in zebrafish. A, Melatonin content of whole-larval extracts; B, Melatonin release by embryos in flow-through culture. See text.

Specific aim 3. Behavioral rhythmicity. We proposed to “develop automated methods for measurement of zebrafish locomotor rhythms and characterize the clock properties of the intact circadian system.” After testing numerous configurations, we developed an infrared detector method for measuring

behavioral rhythmicity from individual adult (6 weeks and older) zebrafish. Fish are placed individually in 50 ml tissue culture flasks modified to accommodate inflow and outflow tubing for aquarium water, which circulates at 10 changes per hour through common biological, fiber, and carbon filters and a UV sterilizer. The flasks are placed in light-tight boxes fitted with a single infrared beam detector for each flask. Beam crossings are counted by a MiniMitter DataCol III program on a PC computer. The entire system is set up in a modified constant temperature room that regulates the water temperature within  $\pm 0.1^{\circ}\text{C}$ .

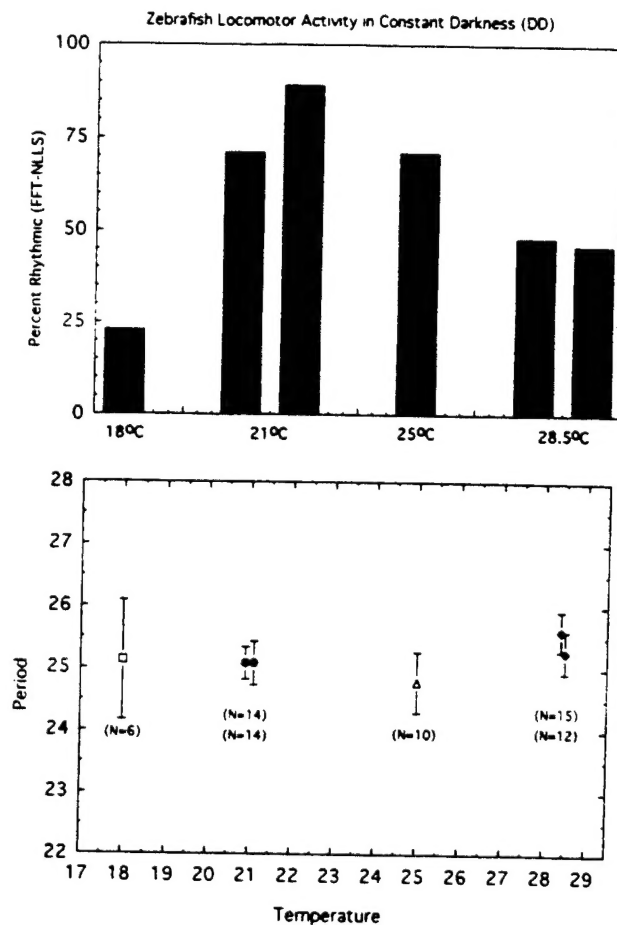
We have recorded locomotor activity from several hundred adult zebrafish, each during 10-14 days in constant conditions. In 95% of AB strain fish, significant (95% confidence level) circadian periodicity can be detected by Monte Carlo- high resolution Fourier analysis, while more conservative methods (periodogram analysis or coupled fast Fourier transform-nonlinear least squares analysis (FFT-NLLS)) detect significant circadian periodicity in a lower proportion of adult fish. The double-plotted actograms in Figure 3 illustrate the range of activity patterns that we observe. Animals were transferred from an LD cycle (indicated at the tops of the figures) to constant conditions on the first day of each record. The number of activity events during each 2 h period was measured, a centered 24 h average was subtracted from each point to remove long-term trends, and the positive values were plotted. This results in a plot of activity during time intervals when the level is above the 24 h mean. Figures 3A-C are from fish in constant dark, and Fig. 3D is from a fish in constant light. Actogram 3A is one of the most stable rhythms that we have recorded, while actogram 3B is typical; both have significant circadian periods by FFT-NLLS. Monte Carlo simulation analysis detects a 22.8 h period in actogram 3C, but FFT-NLLS detects no significant circadian period in this record.

We have tested the effects of temperature and lighting conditions on adult male and female zebrafish rhythms. Temperature affects the strength of rhythmicity, with strongest periodicities measured at 21-25°C (Fig. 4A), but temperature does not affect the periods of rhythms (Fig. 4B). Rhythm characteristics are unaffected by the sex of the fish or whether the previous entraining cycle was LD 12:12 or LD 14:10. In constant light, mean period is significantly shorter at all temperatures, but other characteristics of the rhythms are similar. The great majority of the animals are most active during the projected day phase. A manuscript describing the adult behavioral rhythmicity is in preparation.



**Figure 3.** Actograms of adult zebrafish in constant conditions; see text.

**Figure 4.** Effects of temperature on circadian rhythmicity in adult zebrafish. Top: The probability of individual fish producing strong circadian rhythmicity is temperature dependent, with best rhythmicity at 21-25°C. Bottom: The period of the behavioral rhythm is temperature compensated. Both graphs show data from 2 experiments each at 21° and 28.5°, and one experiment each at 18° and 25°C.





Behavioral rhythms in juvenile zebrafish. We have tested the feasibility of measuring behavioral circadian rhythmicity in juvenile zebrafish by automated video image analysis. We now have direct evidence for robust, precise circadian rhythmicity in juvenile zebrafish during 3-4 days in constant conditions (Fig. 5). Two-week old fish were placed in the wells of a 96-well microtiter plate and 4-week old fish were placed in a 6 well tissue culture plate at the time of light offset. The plates were placed under constant far-red/infra-red light ( $\lambda > 700$  nm) in an incubator at a constant temperature of 24°C. Time-lapse video recordings were made using borrowed equipment. The first day was recorded at a slow tape speed, such that 24 hours are condensed to 2 hours of tape. The last 2.5 days were recorded on 35 minutes of video tape by taking snapshots at 1 second intervals during every other minute. Activity was quantified by visual measurement of the number of times each fish crossed the center line of its well.

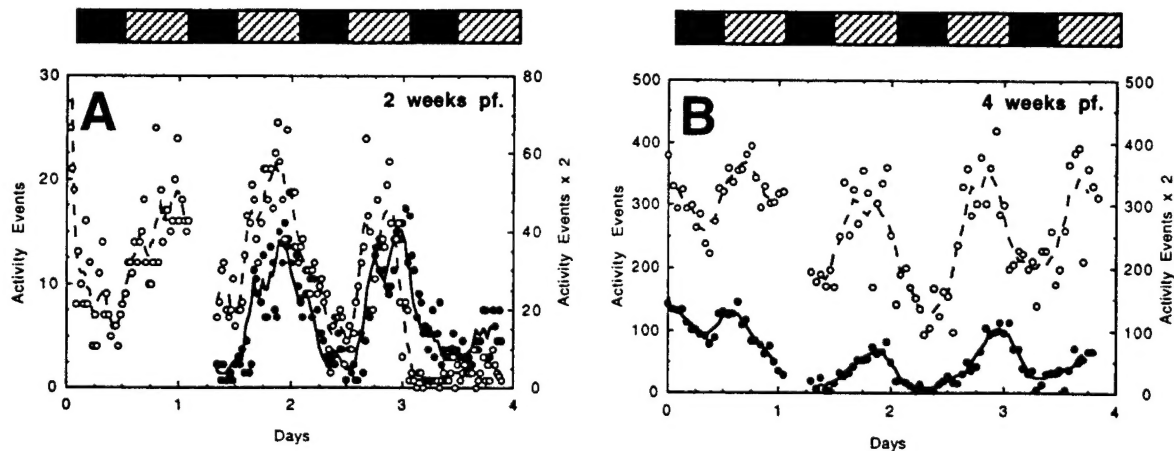


Figure 5. Activity rhythms of 4 juvenile fish measured from time-lapse video recordings. The left y-axis on each graph is for the first day, and right y-axes are for the last 3 days. Lines plot a 5-point centered moving average of the point estimates (30 minute bins in A, 60 minute bins in B).

Specific aim 4. Oviposition rhythms. Zebrafish have been reported to breed within an hour of light onset in a 14:10 LD cycle, suggesting that oviposition is controlled either by a circadian clock or in direct response to the light. In another teleost species, circadian rhythms of oviposition persist in constant light. We proposed to determine for zebrafish whether rhythms of oviposition

persist in constant conditions and whether the timing is controlled exclusively by the circadian system of an individual female or influenced by social interaction. At the time we believed that it might provide a useful measure of rhythmicity. Now, after considerable experience breeding zebrafish, we still think that this would be interesting biology, but not a reliable measure of rhythmicity for genetics or physiology. In our hands, fish kept in a 14:10 LD cycle breed over a 4-5 hour period in the morning, but individuals (and sometimes whole tanks) do not breed every day. Breeding success is very dependent on regular feeding with live food, which creates a major disturbance, and the fish do not breed well when kept at high density. Therefore, we have not pursued this aim.

*Specific aim 5. Rhythms of anesthetic and toxin sensitivity.* We proposed to determine whether a selection strategy based on rhythmic sensitivity to a toxin or anesthetic could be used as a rapid initial screen for potential clock mutants. The sensitive phases of animals with mutant circadian periods would be expected to rapidly drift away from the sensitive phases of wildtypes in constant conditions. Animals resistant to a treatment at times when wild types are susceptible would be potential clock mutants. Animals preselected in this way could then be tested for abnormal behavioral rhythmicity, to determine whether there is a clock mutation.

We have done several experiments to determine whether the susceptibility of larval zebrafish to an anesthetic (tricaine) or an acute toxin (the fungicide, captan) changes with time of day. We found that susceptibility to tricaine was highly variable, with a broad dose-response curve, and have not pursued it as a possible selection agent. It was previously reported that immersion of larval zebrafish in 1 ppm captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboxyimide) results in neurotoxicity, causing excitation initially, immobility within 30 min, and 100% mortality within 90 min. We confirmed that this toxin has a very steep dosage-mortality curve in larval zebrafish, and have found some evidence for rhythmicity in the sensitivity (Fig. 6). In this experiment, 2 groups of 10 day old larvae were entrained 6 hr out of phase with each other, then treated simultaneously to control for temperature and other variables. The data, plotted

observed this multiple times. However, there is a difference in dose dependence between the 2 groups, with the advanced group being generally more susceptible. The variation in dose dependence has been even larger between experiments. In order to be useful in a selection scheme, we will need to define a single dose that reliably distinguishes animals at different phases. Since the source of the variability is not clear, this does not appear to be a useful screening method.

Figure 6. Diurnal variation in sensitivity of larval zebrafish to captan.

